Proceedings of the Iowa Academy of Science

Volume 70 | Annual Issue Article 85

1963

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Return of Pigment in Regenerative Tissue After Inhibition by Phenylthiourea[1]

SISTER FRANCIS CLARE LADD[2]

Abstract. Inhibition of melanogenesis in larval cells of five amphibian species (two genera) was attained by phenylthiourea treatment. Buff-colored granules were demonstrated in cells of three species, gray granules in two. Pigment returned five to fifteen minutes after withdrawal of PTU. The delay is thought to be due to the time needed for the autocatalytic formation of some dihydroxyphenylalanine, which with tyrosinase is required for melanogenesis, as reported by Lerner and Case. Results obtained with living cells were corroborated by study of fixed material. The data support an origin within the Golgi complex or endoplasmic reticulum.

Most of the extensive literature on melanogenesis in animals has been concerned with the morphology and chemistry of melanin formation in mammals. This study concerns melanin formation in larval anurans following inhibition of melanogenesis in both normal and regenerative tissue.

Several classes of compounds are capable of chemical inhibition of melanin formation in animals. Some of these compete with copper in the action of the essential enzyme, tyrosinase; others combine with copper. Among the latter are some derivatives of thiourea which have proved highly effective in amphibia (Lynn, 1948; Millot and Lynn, 1954; Lynn and Dent, 1957). Of these, phenylthioura is probably the most potent. Since melanin formation is essentially an oxidative polymerization, it also may be inhibited by reducing agents such as ascorbic acid.

The results of melanin inhibition are particularly striking when treatment is given to young larvae before the larval pigment has appeared. These individuals do not develop any new pigment as long as the phenylthiourea treatment is continued. Withdrawing the treatment results in reappearance of melanin. Similarly, administration of phenylthiourea to tadpoles during

[1] Taken from a dissertation submitted at The Catholic University of America in partial fulfillment of the degree, Doctor of Philosophy.

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the period of tail regeneration after amputation prevents the appearance of melanin in the regenerate during treatment. In this study the formation of melanin in normal and regenerative larval tissues, after discontinuance of phenylthiourea, has been followed in detail in the hope of obtaining some information as to the site of melanogenesis in the cell.

Materials and Methods

Eggs and tadpoles of *Rana sylvatica, R. catesbeiana, R. palustris, R. pipiens,* and *Bufo americana* used in this study were collected locally; *R. pipiens* eggs were also obtained by induced ovulation. The eggs were separated and placed in shallow white pans of about a liter capacity, and the media were changed daily. Some of the animals were treated with phenylthiourea with no amputation. Others were allowed to grow without treatment for some weeks; their tails were then amputated and the animals were treated with phenylthiourea (hereafter referred to as PTU) during tail regeneration. Concentrations of PTU ranged from 0.01% to 0.001%, since after approximately ten days the concentration could be reduced without any change of results.

Several methods for study of living material were used. Neutral red, 1:10,000 to 1:1000, was applied as a vital stain for fat droplets and for granules described by Greenberg, Kopac and Gordon (1956) as premelanin granules. These, without pigment inhibition would be black in the species studied. With the higher concentrations the premelanin granules pick up the dye more quickly. In some cases the animals were kept in the dye for 24 hours after which they were anesthetized and their tails examined under the microscope. In other cases, freshly excised tissue was mounted on slides and dye drawn under the coverslip. The effect of the dye was the same in all cases: premelanin granules became rust colored; fat globules became yellow.

Aggregates of pigment were located with relation to the nucleus by the use of 45% aceto-orcein drawn under the coverslip on fresh regenerates. The anesthetic used in all cases was MS-222 solution prepared with 0.01% PTU as solvent.

To supplement the studies on living melanophores, sections of tissue fixed at intervals after cessation of PTU treatment were studied. A solution of 7% formalin, made with either water or 0.01% PTU solution as solvent, was tried. Experience showed that the best procedure was the use of the 0.01% PTU as solvent since the use of water alone resulted in some appearance of melanin in the specimen. Tissues were dehydrated in a graded series of alcohols from 35% to 100%, cleared in benzene, embedded in Tissuemat. Since the pieces of tissue were only a few square milli-

meters in size and were practically colorless, they were supported in small wire baskets and colored slightly with neutral red to facilitate handling.

Sections were cut at 8 to 10 mu, mounted serially, and stained in Mayer's or Grenacher's carmalum. Sectioned material was studied with an oil immersion lens, with and without neutral filter; in addition, living material was examined with both a water immersion and an oil immersion phase-contrast objective.

Observations

Pigment Inhibition in Unoperated Larvae. Pigmentation is inhibited by PTU in all five species used in this study. Tadpoles of *R. palustris, R. catesbeiana* and *R. sylvatica* become straw colored because hemoglobin shows through tissue that is now nearly transparent (Fig. 1). *R. pipiens* and *B. americana* tadpoles be-

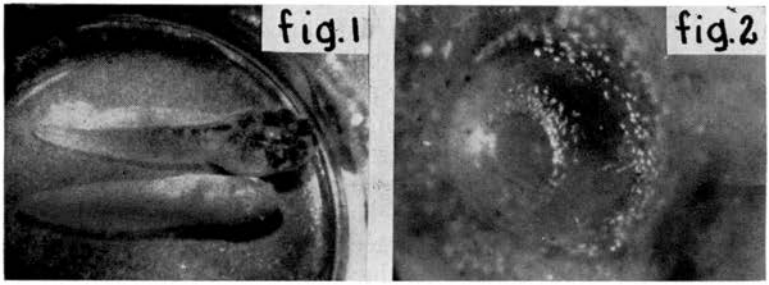


Figure 1. *Rana palustris.* The lower tadpole was treated with 0.005% phenylthiourea for five weeks beginning with the neural groove stage.

Figure 2. *Rana sylvatica.* Eye dissected from tadpole treated with 0.0075% PTU for five weeks beginning with the neural groove stage.

come gray. After several weeks of treatment, gill areas in all become distinctly red; this is true even in the very highly pigmented tadpoles of *B. americana.* In all species pigment is retained longer in the retina than in any other area but eventually most of this is also lost in *R. palustris, R. catesbeiana* and *R. sylvatica* (Fig. 2). Regardless of when the PTU treatment is begun, the whole tadpole gradually pales, and it is possible to see that pigment cells in the tail region have migrated to the tail fin edges where they are eventually no longer visible.

An examination of the anesthetized animal under low power shows still some areas in a few cells wherein pigment granules remain. These are in large, many-branched, otherwise clear cells. In nearly every case, this small, often angular cluster of pigment granules is localized in that part of the cell nearest a blood vessel.

Pigment Inhibition and Reappearance in Regenerating Tail Tissue. In those animals allowed to grow some weeks before the tails were amputated, and kept in PTU during regeneration,

there usually resulted an apparently pigmentless tail on an otherwise near-normal appearing animal. A junctional notch where regeneration began and a more rapidly tapering notochord make the regenerate easy to recognize. Moreover, regenerated tissue contains more fat globules than normal tissue. In those animals treated with mild solutions of neutral red over a 24-hour period, tissue containing more of these globules facilitates its recognition as regenerated tissue.

R. pipiens and *B. americana* differ from the others in some respects. In the early stages of development in *R. pipiens*, pigment cells appear within the regenerating tail. It is likely that these cells have migrated along the notochord into the regenerate. Tail amputation in later stages, however, is followed by melanin inhibition in the regenerate. In *B. americana*, embryonic pigment cells appear within the regenerate, but, with time, successively closer to the edge. A second amputation of the edge alone results in a newly formed tail almost devoid of pigment. Eventually, then, in all cases studied the treatment results in animals with a regenerated, non-pigmented tail.

Microscopically, the contrast in appearance between melanophores of normal, non-pigmented tails and those regenerated under treatment is striking. Melanophores of control animals contain black, round separate melanin granules usually responding to bright light with accelerated movement. Cells of the regenerate in treated animals, on the other hand, contain granules of the same size, but of dull yellow or buff color in three species and gray in two. These granules (Fig. 3) are most often found as aggregates. They are able to take up neutral red (Fig. 4) becoming bright rust red in contrast to fat globules which appear yellow with this dye. These are the previously mentioned premelanin granules. The number of cells containing these is roughly the same per high-power field as is the number with

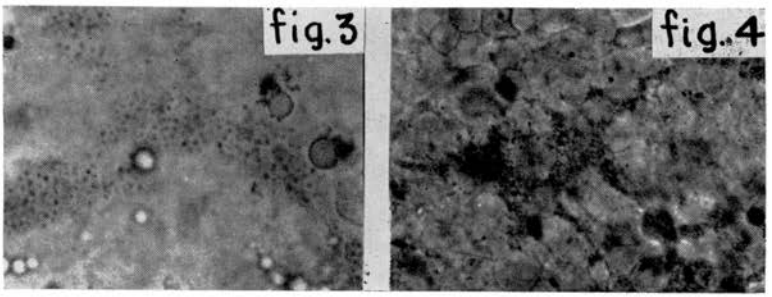


Figure 3. *R. palustris*. Pigment cell within the tail regenerated under treatment. No dye has been used. The propigment granules are contiguous and of a buff color. In this cell they extend throughout the processes. A few fat globules may be seen.

Figure 4. *R. sylvatica*. A similar cell but here the propigment granules have been treated with neutral red. They assume a rust-red color with this dye.

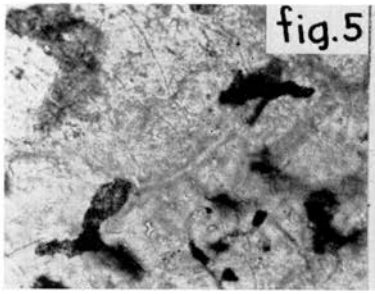
black granules in the controls. In treated tadpoles of *R. pipiens* and *B. americana*, the granules of pigment show varying degrees of gray (Fig. 5), while those in *R. catesbeiana*, *R. sylvatica* and *R. palustris* are buff-colored.

Tissue regenerated during treatment with PTU is especially favorable for the study of the return of pigment after treatment has been withdrawn. This can be observed in tissues fixed at intervals after tap water has been substituted for PTU, or more effectively, in tissue freshly excised and treated with tap water on the slide.

A microscopic examination of fresh tissue preparations mounted in PTU solution reveals that the gradual substitution of water for PTU solution by drawing it under the coverslip causes a darkening of the premelanin granules within a few minutes. Darkening begins at the edge of the granule and proceeds over the whole, changing its color from buff to dull tan, thence to gray. This is best observed, of course, in those species whose premelanin granules assume a dull yellow color rather than a gray appearance, but it is detectable in the gray granules as well.

Tadpoles of the different species studied and even tadpoles developed from different batches of eggs of the same species require varying periods of days in water for complete darkening of melanophores *in vivo*. In freshly excised tissue from treated animals, however, the most noticeable color change occurs after a period of about five minutes and before the end of 15 minutes. After this interval the rate of restoration is considerably reduced. Later, though additional darkening has taken place, it is not so easily detected. A study of sectioned material fixed at five minute intervals, hourly intervals, and daily for several days confirms these observations. It is to be noted that pigment granules in cells of *R. palustris* have an extended brown phase.

In fresh preparations, aggregations of premelanin granules, often adjacent to the nucleus, are clearly noticeable after the addition of tapwater. These apparently correspond to the perinuclear caps of melanin found in untreated larvae and described by Greenberg et al. (1956). The addition of aceto-orcein by drawing it under the coverslip affords a clear delineation of the nucleus and demonstrates its frequent association with these areas of darkening pigment (Fig. 6). Usually there is one perinuclear cap per cell, but occasionally two and less often three are demonstrable with oxygenated water and a nuclear dye. When the cell membrane ruptures during preparation, often by no more harsh treatment than the weight of the coverslip alone, the nuclear caps can be seen floating as units around and between intact cells. In the living animal, the components of the cap are later free to disperse within the cell.



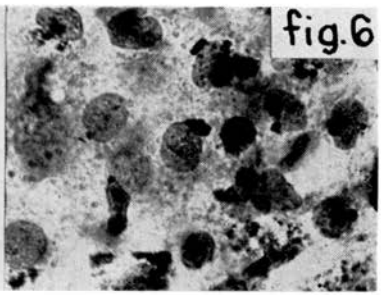


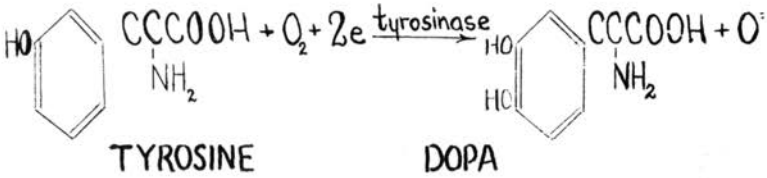
Figure 5. *R. pipiens*. Granules of pigment in cells treated with PTU in this species as well as *B. americana* show varying degrees of gray instead of buff. Neutral red, 1:10,000 has been drawn under the coverslip.

Figure 6. *R. syvatica*. Cells of tail tissue first treated with PTU to inhibit pigment formation. PTU was withdrawn and water substituted for a period of fifteen minutes. This was followed by a nuclaer dye to demonstrate the close association of pigment with the nucleus.

Smaller aggregates of premelanin granules occur in other parts of the cell as well as adjacent to the nucleus. In such cases the nuclear caps may or may not be present.

Discussion

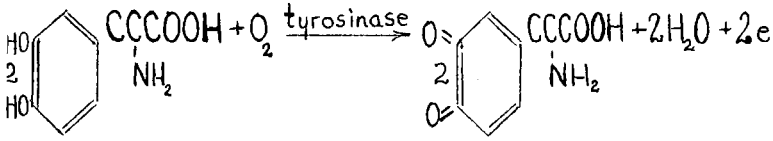
The darkening process in pigment cells, accomplished by the polymerization of tyrosine to melanin, is known to involve the enzyme, tyrosinase. This enzyme, a copper-containing protein belonging to a class of enzymes called the phenolase complex, is the catalyst in two successive reactions. That substance formed in the first reaction is dihydroxy-phenylalanine (DOPA); its formation involves molecular oxygen as in the following equation.



Before the DOPA of this reaction can be formed, however, two electrons are needed. These are provided when a trace of DOPA is formed autocatalytically as was shown by Lerner and Case (1959) by the use of O^{18} tracer. One atom of the oxygen molecule in inserted into the ring forming the diphenol; the other is reduced ultimately to form water. DOPA, then, is the electron donor, and must be present to start the hydroxylation process. In its absence, an induction period results which ends when some is formed autocatalytically. Once the reaction begins new DOPA is formed as fast as it is used up.

In the second reaction, DOPA is oxidized to o-DOPA-quinone

during the formation of which water and two electrons are released.



DOPA o-DOPA-QUINONE

Thus, an animal is able to obtain from tyrosine large amounts of DOPA-quinone without any necessity for maintaining a large amount of DOPA which is toxic and has reducing properties.

The steps following DOPA-quinone formation as proposed by Lerner and Case (1959) include the formation of a leuco-compound, dopachrome, 5,6-dihydroxyindole, 5,6-indolequinone and finally its polymerization to DOPA-melanin through quinone linkages.

The presence of two hydroxyl groups in the 3,4 position of the phenyl nucleus is essential for the positive production of melanin within melanophores (Burgers, 1959). Conversely, positive inhibition of pigment formation results from the removal of the alpha-amino group or substitution of a methyl radical for the alpha-hydrogen (Wilde, 1955). For polymerization to proceed, the steric configuration must be preserved (Wilde, 1959).

This study shows that when PTU is withdrawn from cells which have undergone melanin inhibition and is replaced by oxygenated water, a color change occurs within a period of about five minutes. This period corresponds to the time needed for the above-mentioned autocatalysis of DOPA. Once a trace of this compound is formed, further darkening proceeds rapidly and visibly. It is this undesired reaction that necessitates the use of PTU solution as solvent with reagents such as dyes and anesthetics when inhibition of melanin is to be maintained. Because this reaction proceeds even in fixed material, PTU solution must also be used as solvent for fixatives if inhibition is to be maintained.

The premelanin granules of this study are thought to represent the protein (Greenberg, Kopac and Gordon, 1956) or pseudoglobulin (Kanof, 1955) matrix required for polymerization of melanin since a melanin-protein complex is essential. This corresponds to what Weissenfels designated as precursor promelanin in granules from which melanized particles arise and to which Renyi (1924) refers as *Pigmentbildner.*

The origin of the melanin granules within the cell has been

the subject of considerable study. The nucleolus (Mierowsky, 1906) and chromatin (von Szily, 1911) have been reported to be the site of origin in earlier studies. Later studies have been centered around the mitochondria, Golgi complex and the endoplasmic reticulum. Information concerning these subcellular particles is supplied by biochemical data and electron microscopy.

Some biochemical evidence favors a mitochondrial origin of pigment. Mammalian tyrosinase was reported by Lerner (1953) and Levin (1956) to be located on the mitochondria and by Lerner (1959) to be bound to melanin granules. This is in contrast to plant tyrosinase which is soluble. Other reactions (Greenberg et al. 1956; Becker, 1952) show the concentrations of this enzyme to be greatest in the area where the nuclear caps appear. As found in this study, the early and frequent presence of these caps of melanin as well as their appearance after PTU has been withdrawn, substantiates this juxtanuclear placement of this enzyme.

A preponderance of evidence, physical as well as biochemical, is against the mitochondrial origin of melanin granules, however. These two particulates react differently to acids: melanin granules are stable in acid solutions, mitochondria are not. Within the cell, melanin granules move in a jerky manner whereas mitochondria drift and move more slowly. If mitochondria become pigment granules, their number would be expected to decrease as the number of granules increases; this decrease was not apparent.

Further evidence that mitochondria and melanin granules are two distinct cytoplasmic constituents of the melanin-forming cell is also provided by density gradient centrifugation (Baker et al., 1960). This method places these particulates in different density layers. When this separation is followed by a biochemical study of the resulting fractions, no tyrosinase activity is found in the mitochondrial layer; but, as might be expected, 68% of the succinoxidase activity is here recovered.

The ultrastructure as provided by the electron microscope corroborates biochemical distinction between mitochondria and pigment granules. The developing melanin granule as seen in the electron microscope has, in addition to a double external membrane (Dalton, 1959; Moyer, 1961), internal membranes in both concentric and crumpled spiral arrangement; this is in sharp contrast to the typical arrangement of cristae within the mitochondria. Though initially these membranes are of the same density as other membrane structures of the cell (i.e., Golgi, nuclear, mitochondrial membranes), later they thicken considerably so that the mature granule is uniformly dense and apparently struc-

tureless. The varying rates of melanin development in these membranes may explain the varying rate of darkening of premelanin granules developed in the cells used for this study; first rapidly, then slowly over a period of days.

In view of the association of the synthesis of protein with the presence of an endoplasmic reticulum, it is not suprising that a well-developed endoplasmic reticulum is found in melanocytes. Such a reticulum is reported by Dalton (1959) Wellings and Siegel (1959), Birbeck and Barnicot (1959), and Moyer (1961). Moyer demonstrated a developmental sequence of four stages for melanin granules with granules of stage one continuous at several points with cytoplasmic membranes which he tentatively identifies with the endoplasmic reticulum. He did not associate these membranes with the Golgi complex in any way.

Wellings and Siegel (1959) believe a minority of granules might originate within membrane-enclosed spaces of the endoplasmic reticulum, particularly since occasionally the reticulum may be contiguous with the Golgi membranes. These authors favor a Golgi origin, however. The endoplasmic reticulum is considered by them not to be the site of origin because melanin granules were never observed within spaces enclosed by membranes whose outer surfaces were provided with ribonucleoprotein particles.

Dalton (1959) found the unique system of membranes within the melanocyte to be distinct from both the Golgi complex and the endoplasmic reticulum. He believes precursors of melanin granules are formed by the enlargement of Golgi vesicles.

The main evidence that the Golgi complex is involved is a spatial, intracellular relationship between the immature granules and the Golgi vesicles (Birbeck and Barnicot, 1959), and in turn the relationship between these premelanin granules and fully melanized ones. The appearance of granules in the various stages of development in the large vesicles suggest that these are the sites of accumulation of the dense pigment (Birbeck, Barnicot and Mercer, 1956; Dalton, 1959).

The same juxtanuclear position within the cell of the Golgi complex and the perinuclear caps of darkening pigment found in this study add more substantiation to a Golgi origin of melanin granules. However, despite the frequent presence of nuclear caps, aggregates of pigment were seen to develop as often in other areas of the cell.

It is possible that the site of formation of visible melanin granules and the site of the synthesis of molecular melanin in the pregranule stage are not identical. The completion of the mature melanin granule probably requires the participation of several

cell organelles, whether the evidence is interpreted as favoring the conclusion that the granule has its origin in the Golgi complex or in the endoplasmic reticulum.

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